THE IMPORTANCE OF FORMIC DEHYDROGENASE IN THE OXIDATION MECHANISMS OF PISUM SATIVUM.

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(Two Text-figures.)

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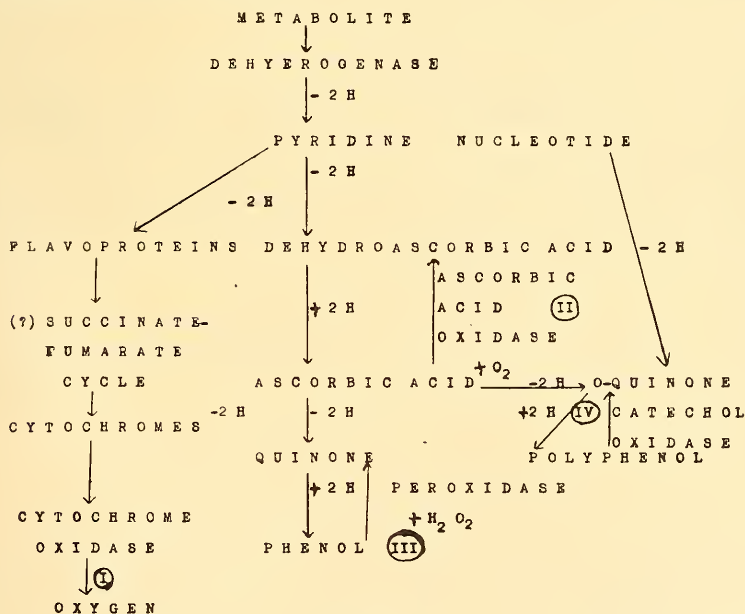
INTRODUCTION.

The widespread occurrence of the enzyme formic dehydrogenase in plant seeds has been reported in a previous paper (Davison, 1949). Any attempt to understand the position of this enzyme in the metabolism of the plant must include a study of the respiratory mechanisms to be found. The oxidative chain reactions already postulated for plants are summarized in Scheme I.

As can be seen from this scheme, there are three main terminal oxidases and one peroxidase and at least six paths by which biological oxidation could proceed. Attempts have been made to find out which of these paths can be used in the breakdown of formic acid to carbon dioxide and water in the pea seed. In order to make the results more complete and perhaps discover some significant changes in activity, the enzymes of the seven-day seedling were also studied.



SCHEME I

MATERIALS AND METHODS.

The peas used were dried peas bought in 1 lb. packets and soaked overnight before use when they took up their own weight of water. Various methods were used for the preparation of the extracts depending on the nature of the enzymic relationships and the conditions necessary for their demonstration. These methods are described in their appropriate places. The seven-day-old seedlings were grown on damp filter paper in an

enamel tray under a glass cover. The whole seedling was used in the preparation of extracts which were made in the same way as the corresponding seed extract.

The Thunberg technique was followed in anaerobic experiments on dehydrogenases and a variation of this technique in work on ascorbic acid-formic dehydrogenase coupling. Oxygen uptake was measured in Warburg manometers fitted with flasks carrying two side arms; the volumes were between 20 and 25 ml. The manometers were shaken at 102 cycles per minute in a constant temperature water bath at 38°C. Usually the final volume of each flask was 3·2 ml. To absorb CO_2 produced, 0·2 ml. 20% NaOH was placed in the centre well, together with a KOH-paper made of Whatman No. 40 filter paper as suggested by Dixon (1943).

Coenzyme I was prepared by the method of Williamson and Green (1940) from yeast, and its dihydro derivative estimated spectrophotometrically by absorption at 340mμ. Sodium pyruvate was prepared by the procedure of Robertson (1942) and estimated manometrically by the production of CO_2 by enzymatic decarboxylation (Westerkamp, 1933). Other chemicals were of reagent grade purity and were neutralized or brought to the appropriate pH before use. Buffers and the pH of flask contents were checked frequently by means of a Leeds and Northrup portable glass electrode.

RESULTS.

DEHYDROGENASES AND CARRIER-LINKED DEHYDROGENASE REACTIONS.

In the first instance formic dehydrogenase must be regarded simply as one of the many dehydrogenases to be found in the pea. In this connection the effect of a large number of substrates has been tested anaerobically in Thunberg tubes and aerobically in manometers.

In anaerobic studies peas were soaked overnight, homogenized for two minutes under nitrogen in a Waring Blendor with an equal volume of water, squeezed through muslin and the extract centrifuged for 5–10 minutes to remove starch. The green cloudy supernatant fluid was 80% saturated with solid $(NH_4)_2SO_4$, the sediment taken up in water, the solution centrifuged for 15 minutes and the supernatant used. Substrates which were found to reduce the decolorization time include formate, alcohol, malate, fumarate, glutamate and lactate (Table 1). As a rule succinate could only be included in this list when an untreated, lightly centrifugd water extract was used. This is not surprising since succinic dehydrogenase is not soluble in distilled water. Substances which either made no difference to the decolorization time or else inhibited the reduction of the dye were glucose, citrate and glycerophosphate.

TABLE 1.

Dehydrogenases Present in Pea Seeds and Seven-day Old Seedlings.

Tube contents: 1·0 ml. extract, 0·5 ml. 0·2 M phosphate buffer pH 6·4, 0·2 ml. coenzyme I (60μg.), 0·3 ml. substrate (final concentration 0·02 M), 0·2 ml. 0·0025 M thionine.

Substrate.	Decolorization Time. (Minutes.)	
	Seed Extract.	Seedling Extract.
Water	65	51
Formate	7	16
Alcohol	4	45
Malate	16	14
Fumarate	18	15
Glutamate	30	32
Lactate	40	42
Succinate	34	35
Glucose	65	51
Citrate	62	50
β-Glycerophosphate ..	67	51

In the seven-day seedlings there were only two marked changes in the dehydrogenase content as measured by the Thunberg technique and correlated on the basis of dry weight. The activity of both alcohol and formic dehydrogenases had fallen off, the former considerably (Table 1).

In both preparations it was interesting to note that, when malate and fumarate were substrates, the time of incubation before the dye was tipped was an important factor in comparing the decolorization times. The longer the period of incubation (generally 15–20 minutes were enough) the more closely the times agreed. This was due no doubt to the fact that the enzyme fumarase has first to add water to the fumarate to form malate before it can be dehydrogenated by malic dehydrogenase.

Of the dehydrogenase found, formic, malic, alcohol, glutamic and lactic all required the presence of coenzyme I before reduction of thionine would take place. Possibly the negative result with citrate was due to lack of coenzyme II (necessary for the working of isocitric dehydrogenase). However, citrate failed to reduce the decolorization time even in the untreated water extract which, presumably, contains some coenzyme II (van Herk, 1935).

For the aerobic experiments a water extract of peas was used after centrifuging for 10 minutes to remove starch and cellular debris. Methylene blue was used as hydrogen carrier in these manometric experiments and a pH of 6·4 was chosen since that is the natural reaction of pea juice and of water extract of peas. The results of a typical experiment using seed extract are seen in Table 2.

TABLE 2.

Effect of Added Substrates on Oxygen Uptake of Pea Seed Extract.

Manometer contents: 1·6 ml. extract, 0·8 ml. 0·2 M phosphate buffer pH 6·4, 0·2 ml. coenzyme I (60 μg.), 0·3 ml. 0·2 M substrate, 0·1 ml. 0·5% methylene blue, 0·2 ml. 20% NaOH in centre well.

Substances which Increase the Endogenous O_2 Consumption.	μl. O_2 in 60 Mins.	Substances which have No Effect or which Decrease the Endogenous O_2 Consumption.	μl. O_2 in 60 Mins.
Water	85	Lactate	79
Formate	164	Oxalate	83
Alcohol	156	Glycerophosphate	85
Malate	118	Formaldehyde	75
Glucose	127	Acetaldehyde	86
Fumarate	120	Acetate	85
Succinate	97	Pyruvate	59
Glutamate	138	Citrate	77
Glycine	122	Sucrose	83
		Tyrosine	80
		Catechol	69
		m-Cresol	77
		Phenol	74

In aerobic experiments using seven-day seedlings the O_2 uptake of the blank endogenous respiration was higher but the effect of the various substrates was much the same as for seeds, except that, in the seedling, sucrose, lactate and citrate can be included in the list denoting increases in oxygen uptake (Table 3).

Carrier-linked Reactions.—Experiments were next made to determine whether the formic dehydrogenase could be linked with any of the other dehydrogenase systems found in the pea seeds. Of these three were immediately eliminated because of the fate of their reaction product. Both oxalacetate and pyruvate, the substances resulting from dehydrogenation of malate and lactate, are immediately decarboxylated by pea extract. In the case of glutamic dehydrogenase, the reversibility of the reaction, glutamic acid + O ⟶ α-keto-glutaric acid + NH_3, is difficult. There remained the possibility of coupling

TABLE 3.
Effect of Added Substrate on Oxygen Uptake of Seven-day Seedling Extract.
Flask contents as in Table 2.

Substances which Increase O_2 Uptake.	µl. O_2/hr.	Substances which Decrease O_2 Uptake.	µl. O_2/hr.
Water	183	Oxalate	180
Formate	239	β-Glycerophosphate ..	183
Alcohol	200	Formaldehyde	178
Malate	197	Acetaldehyde	185
Glucose	199	Acetate	180
Fumarate	202	Tyrosine	177
Succinate	195	Pyruvate	169
Glutamate	214	Catechol	179
Glycine	212	Phenol	175
Sucrose	190		
Lactate	194		
Citrate	192		

with either succinic dehydrogenase and fumarate or alcohol dehydrogenase and acetaldehyde. Succinic dehydrogenase will dehydrogenate succinic acid to fumaric acid only if an acceptor of hydrogen is present. If, however, activated hydrogen is supplied, for example in the form of dihydrocozymase from formate dehydrogenation, and there is no acceptor like methylene blue present, then one would expect the reaction to go in the other direction. In the case of alcohol dehydrogenase in the absence of methylene blue or other substances which oxidize the reduced coenzyme I, the equilibrium position of the reaction, C_2H_5OH + coenzyme I $\rightleftharpoons$ CH_3CHO + reduced coenzyme, is far to the left (Euler, Adler and Hellström, 1936).

For these coupling experiments an untreated water extract of pea seeds was used after light centrifuging (5–7 minutes). The supernatant therefore contained formic, alcohol and succinic dehydrogenases. Briefly, the principle of the method used was that if one employs a bicarbonate buffer in an atmosphere of CO_2, CO_2 released by the enzymic reactions taking place will escape into the atmosphere and can be measured manometrically. If a coupling of dehydrogenases does occur this will be indicated by increased formate oxidation, and hence increased CO_2 output, in the presence of either acetaldehyde or fumarate. A wide range of pH values were used in these experiments. A typical result at pH 6·4 is presented in Table 4.

It can be seen from this experiment that here there are two carrier-linked reactions in each case, the carrier being reduced by the negative system, formate plus formic dehydrogenase and reoxidized by the positive system, fumarate plus succinic dehydro-

TABLE 4.
Carrier-linked Dehydrogenases.
Flask contents: 1·9 ml. supernatant, 0·3 ml. 0·023 M $NaHCO_3$, 0·2 ml. coenzyme I (60 µg.), 0·3 ml. 0·2 M sodium formate or 0·3 ml. water in one side bulb. In the other side arm was 0·3 ml. 0·05 M acetaldehyde, 0·3 ml. 0·2 M fumarate or 0·3 ml. water. The gas phase was 5% carbon dioxide in nitrogen.

µl. CO_2 produced in 90 minutes.

	Water.	Formate.	CO_2 Due to Formate Oxidation.
Blank	90	102	12
Fumarate	115	155	40
Acetaldehyde	95	150	55

Note.—Acetaldehyde is extremely volatile and on all occasions boiled extract controls were done. These figures are corrected for the boiled extract.

genase on the one hand, acetaldehyde plus alcohol dehydrogenase on the other. Coenzyme I is the only carrier needed in the case of the alcohol enzyme. With the succinic dehydrogenase flavoprotein may, and probably does, also function as an intermediate.

These coupled reactions are only two of the many possible, e.g., between the lactic and malic enzymes as negative systems and the above two, alcohol and succinic dehydrogenases, as the positive systems. Such coupled reactions must play a big role in the economy of the pea seed and the fact that formic dehydrogenase can take part in them increases the importance of this enzyme as a factor in the first step in biological oxidation in the seed.

INTERMEDIATE HYDROGEN CARRIERS.

FLAVOPROTEIN AND THE DICARBOXYLIC AND TRICARBOXYLIC ACID CYCLES.

The next step in the study of formic dehydrogenase as related to oxidation mechanisms was to examine pea extract for the presence of any intermediates or terminal oxidases of Scheme I, and then to establish the relation of formic dehydrogenase to any of the hydrogen carrier systems thus found. The enzyme preparation used for these studies was generally a fine homogenate from which starch and cellular debris had been removed by light centrifuging. Such a preparation contains much insoluble material and particulate matter, including insoluble enzymes plus soluble enzymes and a solution of the freely soluble diffusible components such as inorganic ions, coenzymes and carriers. In addition the energy reservoir is maintained and the study of reaction mechanisms which involve a number of co-ordinated enzymes is possible.

Flavoprotein.—A crude flavoprotein preparation was made from 12-day pea seedlings by the method of Lockhart (1939), but this preparation contained too much formic dehydrogenase to be used in experiments coupling formate oxidation with flavoprotein. Attempts to purify it further by adsorption on C γ alumina only led to its destruction, because of the unstable nature of plant diaphorase (Lockhart, 1939). Soluble diaphorase was therefore prepared from pig's heart (Straub, 1939) and estimated by means of its absorption at 451mμ. The results of experiments with water extracts of pea seeds and seedlings using this diaphorase preparation are seen in Table 5.

TABLE 5.
Effect of Flavoprotein on Formate Oxidation.

Flask contents: 1·2 ml. enzyme, 0·8 ml. 0·2 M phosphate buffer pH 6·4, 0·2 ml. coenzyme I (60 μg.), formate (0·3 ml. 0·2 M solution) and 0·5 ml. flavoprotein (5 μg. bound lactoflavin) as indicated, 0·2 ml. 20% NaOH in centre well, water to 3·2 ml.

	μl. O_2 Uptake in 60 Minutes.		Increase.
	Alone.	Formate.	
Seed Extract—			
Blank	48	58	21%
Flavoprotein ..	52	75	44%
Seedling Extract—			
Blank	110	109	—
Flavoprotein	115	182	57%

Although flavoprotein does not markedly influence the blank O_2 consumption, it has a stimulating effect on formate oxidation especially in the seedling. It can be concluded then that, in the seedling particularly, the oxidation of any formate present can go through this carrier.

Dicarboxylic and Tricarboxylic Acid Cycles.—Malic and citric acids are of common and perhaps universal occurrence in plant tissues. Succinic, fumaric (Pucher and Vickery, 1942) and isocitric acids (Krebs and Eggleston, 1944) have also been found. The critical biochemical evidence for the occurrence of oxalacetic acid in plants rests

ultimately on Virtanen and Laine's (1939) isolation of its oxime from legumes and their nodules and the conversion of the oxime (oximinosuccinic acid) to aspartic acid by reduction. However, no evidence has yet been obtained that these acids take part in a Krebs or Szent-Györgyi cycle.

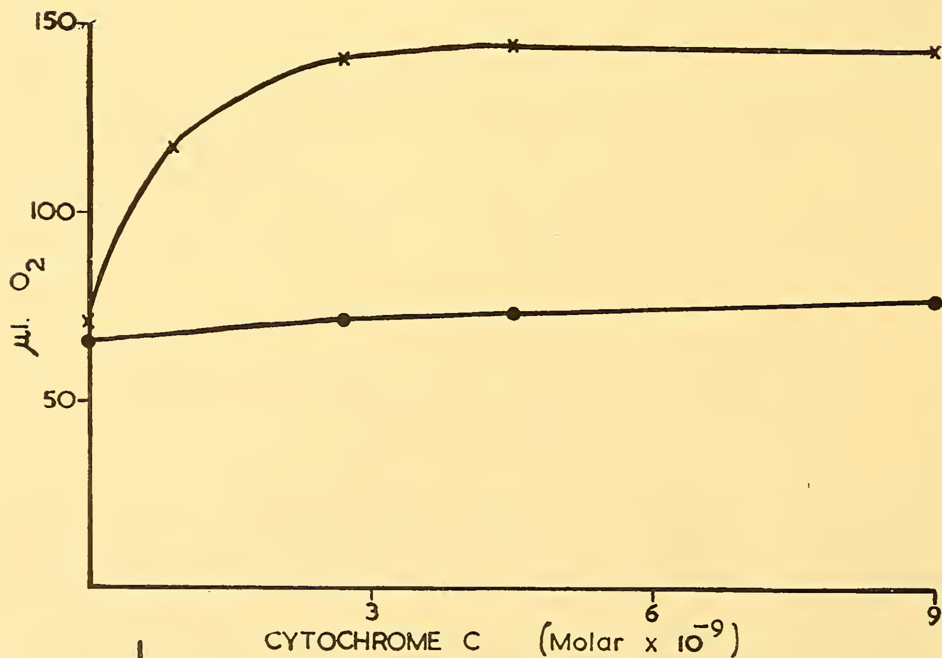
In peas, as mentioned above, malate, fumarate and succinate increase the oxygen uptake of pea extract but, so far from the acids acting catalytically, the extra oxygen consumption was equivalent to only a fraction of the acid added. Citrate was found to have no effect on seed extract. The effect of these acids on formate oxidation by pea extract was measured manometrically at pH 6·4. The results are shown in Table 6, from which it appears that citrate increases formate oxidation more than the other added substrates. Malate and fumarate also have very marked effects but succinate has no effect on the oxidation of formate as revealed by the oxygen consumption.

TABLE 6.

Effect of Acids on Formate Oxidation in Seeds.

Flask contents: 1·4 ml. water extract, 0·8 ml. 0·2 M phosphate buffer pH 6·4, 0·2 ml. coenzyme I (60 μg.). Various additions of C_4 acids, citrate, formate and water were in the side arms, 0·2 ml. 20% NaOH in the centre well.

	μl. O_2 Uptake in 60 Minutes.		Change.
	No Formate.	Formate.	
Blank	53	67	26% increase
Malate	49	73	59% ,,
Fumarate	47	72	52% ,,
Succinate	60	57	4% decrease
Citrate	36	61	70% increase



Text-fig. 1.—The cytochrome saturation value for the formic dehydrogenase system of pH 7·0 buffer extract of pea seeds. Manometer flask contents as for Table 9. The cytochrome is stated as final concentration. x......x, formate as substrate; ●......●, water (blank).

In the seven-day seedling the effect on formate oxidation is not so great, possibly because of the much lower activity of the formic dehydrogenase in seedlings (Table 7).

In the pea seed and seedling, then, although there is no evidence that the C_4 acids and citrate act catalytically there is some indication that they hasten formate oxidation.

TABLE 7.
Effect of Acids on Formate Oxidation in Seedlings.
Flask contents as for Table 6.

	μl. O_2 Uptake in 60 Minutes.		Change.
	No Formate.	Formate.	
Blank	147	153	4% increase
Malate	148	159	8% ,,
Fumarate	149	161	8% ,,
Succinate	150	142	5% decrease
Citrate	131	142	9% increase

TERMINAL OXIDASES: CATECHOL OXIDASE, CYTOCHROME OXIDASE, ASCORBIC ACID OXIDASE AND PEROXIDASE.

Catechol Oxidase.—Although this enzyme has been found in the nodules of peas (Keilin & Smith, 1947), no evidence could be obtained in the present investigations that it occurred in the seeds. No colour was obtained with catechol or α-naphthol, and none of the substrates tested (Table 8) increased the oxygen uptake of an untreated buffer extract. The extract was prepared by homogenizing 25 gm. peas (dry weight) in a Waring Blendor under nitrogen in the usual way, 50 ml. 0·07 M phosphate buffer at pH 7·0 being used instead of water. The supernatant was used after centrifuging for seven minutes.

TABLE 8.
Catechol Oxidase in Pea Seeds.
Flask contents: 2·0 ml. supernatant, 0·3 ml. 0·5% gelatin, 0·3 ml. substrate (2 mg.), 0·2 ml. 20% NaOH in the centre cup.

Substrate.	μl. O_2 Uptake in 45 Minutes.
Blank	59
Catechol	49
Phenol	53
m-Cresol	55
Tyrosine	57

Finally, injured seed tissue does not discolour on standing as is usual in plants containing phenolases (and as was found in making extracts of nodules).

Similar experiments were made with seven-day-old pea seedlings again with negative results so that it can be concluded that there is no catechol oxidase present, a decision which eliminates two of the paths in Scheme I as applied to formate oxidation in peas.

Cytochrome System.—Hill and Bhagvat (1939) have found the four characteristic absorption bands of cytochromes, a, b, and c in the embryos of legumes as well as other plant tissues. Shaking with air caused the bands to disappear, succinate caused reappearance, and reoxidation was prevented by the presence of cyanide and azide. Cytochrome c from ox heart was rapidly reduced by the ground-up plant tissue poisoned with cyanide.

In studying the effect of cytochrome c on formate oxidation the extract was made in the same way as that used for catechol oxidase experiments, i.e., a pH 7·0 buffer extract. The cytochrome c was prepared from ox hearts by the method of Keilin and Hartree (1937) and estimated spectrophotometrically by absorption of reduced and oxidized cytochrome c at 550mμ. The results of experiments on pea seeds using this preparation are seen in Table 9. The amount of cytochrome used is taken from Text-figure 1 and is well over the saturation concentration for seed extract.

TABLE 9.
Effect of Cytochrome c on Formate Oxidation in Seeds.
Flask contents: 1·5 ml. extract, 0·2 ml. coenzyme I (60μg.); 0·2 ml. 20% NaOH in centre well; in the side bulbs, 0·3 ml. 0·2 M formate, 0·3 ml. water or 0·5 ml. $2 \cdot 7 \times 10^{-7}$ M cytochrome as indicated below. Total volume 3·2 ml.

	μl. O_2 Uptake in 50 Minutes.
Blank	68
Formate	70
Cytochrome *c*	89
Formate and cytochrome *c*	165

Results of this type show that cytochrome c accelerates the endogenous O_2 uptake of pea extract and that in the presence of formate this increase is much greater, indicating that the cytochrome system is being used for formate oxidation.

Seedlings were prepared in the same way, i.e., a pH 7·0 buffer extract, and the results are presented in Table 10.

TABLE 10.
Effect of Cytochrome c on Formate Oxidation in Seedlings.
Flask contents as for Table 9.

	μl. O_2 Uptake in 50 Minutes.
Blank	210
Formate	202
Cytochrome *c*	216
Formate and cytochrome *c*	234

These results show that cytochrome c has far less effect on the O_2 uptake of seedling extract than on that of seed extract.

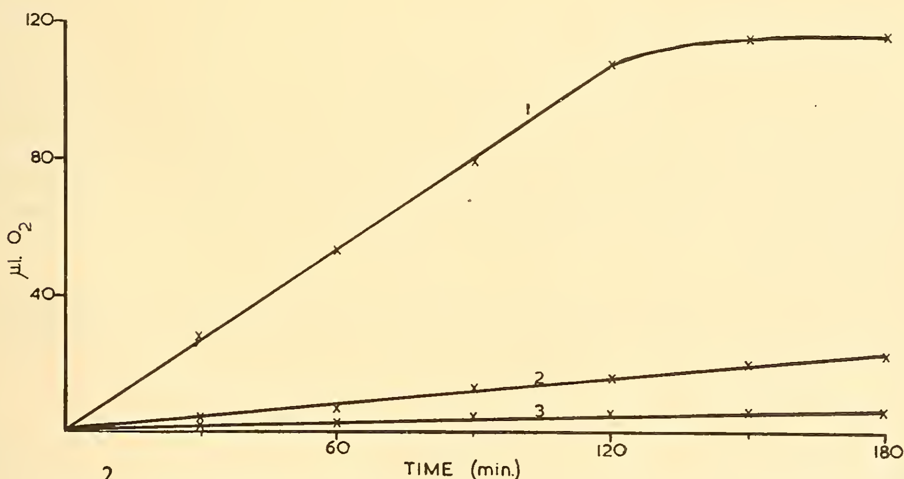
Ascorbic Acid Oxidase.—Although peas before germination do not contain any ascorbic acid, this substance begins to appear immediately upon soaking the dried seed. The following table, taken from Harris and Ray (1933), gives the data on the synthesis of ascorbic acid during germination.

The oxidation of ascorbic acid to dehydroascorbic acid is reversible and Szent-Györgyi's suggestion (1930) that it might behave as a redox body in plant respiration has more recently been substantiated by the discovery of a mechanism for its reduction. James and Cragg (1943) have shown that barley saps catalyse the oxidation of lactic to pyruvic acid by means of the ascorbic system. Later James, Heard and James (1944) established a similar link between the ascorbic acid system and an important intermediate of glycolysis, hexosediphosphate, this time with the hydrogen transfer taking place between coenzyme I and ascorbic acid. It was desirable to find out whether coupling of this latter type could be demonstrated in pea seeds.

TABLE 11.
Synthesis of Ascorbic Acid During Germination.

Material: Seed Peas.	Ascorbic Acid Content.	
	mg./gm.	µg./Seed or Seedling.
Before germination	0·00	0·00
Soaked 24 hours	0·08	0·02
48 hours (germinated)	0·69	0·21
72 hours (germinated)	0·82	0·26
96 hours (germinated)	0·86	0·27

Preliminary experiments designed to show the presence of ascorbic acid oxidase in pea seeds were done on an ammonium sulphate precipitate (Text-fig. 2). The Waring Blendor could not be used in this preparation because the oxidase was inactivated in the process. Peas were ground up in a mortar with sand and twice their dry weight of water. The enzyme was precipitated from the centrifuged extract by 83% saturation with solid ammonium sulphate and the sediment was taken up in one volume of water, dialysed overnight against distilled water and filtered through kieselguhr. This method precluded the occurrence of any cytochrome oxidase in the preparation so that any enzymic oxidation of ascorbic acid observed was due to ascorbic acid oxidase and not the cytochrome–cytochrome oxidase system (see Stotz, Harrer, Schultze and King, 1937). Special precautions were taken to ensure the purity and freedom from copper of all glassware and chemicals used in these experiments. Triple glass distilled water was always used.



Text-fig. 2.—The presence of ascorbic acid oxidase in pea seeds. Manometer flask contents: 1·2 ml. enzyme preparation, 1·5 ml. 0·07 M phosphate buffer pH 6·0, 0·3 ml. water or 0·3 ml. ascorbic acid solution (0·01 mM$\equiv$112 µl. O_2), 0·2 ml. 20% NaOH in centre cup.
Curve 1: ascorbic acid. Curve 2: water. Curve 3: ascorbic acid plus boiled enzyme.

Identical experiments done on the pea seedling revealed a more active ascorbic acid oxidase than in the seed. In the latter 0·01 in M ascorbic acid was oxidized in 130 minutes. In a seedling preparation, corrected for the same dry weight, the time taken was 90 minutes. This increase in activity can be correlated with the increase in substrate concentration in the seedling. As quoted from Harris and Ray (1933), there

is 0·02 mg. ascorbic acid per seed as used after soaking for 24 hours but 0·27 mg/seedling after 96 hours' germination.

From these preliminary experiments the presence of a specific ascorbic acid oxidase in seeds and seven-day seedlings was confirmed. Further experiments, designed to demonstrate the coupling of formic dehydrogenase and this ascorbic acid system, were of two types, one aerobic the other anaerobic. In both cases the pea seeds or seedlings were ground up in a mortar with twice their dry weight of Cu-free M/10 phosphate buffer at pH 6·0. The extract was centrifuged for 15 minutes and the supernatant used.

Although formate by itself, with no carrier, caused little increase in the O_2 uptake of the buffered extract of seeds, substantial increases were obtained on addition of formate to ascorbic acid plus extract. To ensure that the increased oxygen uptake in the presence of formate and ascorbic acid was not due to increased loss of ascorbic acid rather than increased loss of formate, ascorbic acid was estimated at the end of each experiment by titration with 2:6 dichlorphenol indophenol in acid solution (Harris and Olliver, 1942). The results of a typical aerobic experiment are shown in Table 12.

TABLE 12.

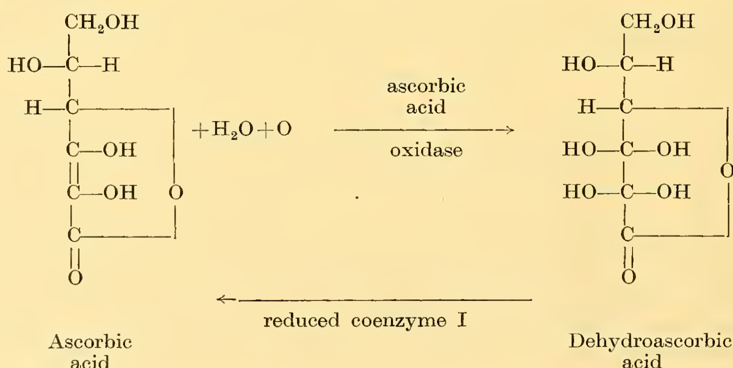
Effect of the Ascorbic Acid System on Formate Oxidation.

Flask contents: 2·0 ml. enzyme preparation from pea seeds, 0·2 ml. coenzyme I (120 μg.), 0·3 ml. water or 0·3 ml. ascorbic acid (0·01 mM) in one side arm, 0·3 ml. water or 0·3 ml. 0·2 M sodium formate in the other side arm, 0·2 ml. 20% NaOH in centre well.

	μl. O_2 Uptake in 60 Minutes.	Total Loss of Ascorbic Acid. (mg.)
Blank	56	—
Formate	59	—
Ascorbic acid	87	0·57
Ascorbic acid + formate	129	0·60

This experiment is typical of many and, in all cases, in the presence of ascorbic acid and formate, there were increases in oxygen uptake over and above that caused by ascorbic acid alone. In most experiments these were not accompanied by any increased loss of ascorbic acid and even if some increased loss were observed (as in Table 12), it was not equivalent to the extra oxygen consumed. For example, the increase of 0·03 mg. ascorbic acid lost here would only entail an increased oxygen uptake of 2 μl., a negligible quantity.

It seems clear then that the increased oxidation (hydrogen loss) is finally borne by the formic acid and not by ascorbic acid. That is, the ascorbic acid system is acting as a carrier in formate oxidation. The chain of reactions responsible for formate oxidation here seems to be:



The reduced coenzyme is produced during the formate oxidation and after being reoxidized by the dehydroascorbic acid is again ready to be reduced by formate.

A buffer extract of seedlings gave evidence of a very active ascorbic acid oxidase and, as with seeds, formate oxidation is increased by the ascorbic acid system although the effect was not so marked probably because of the lower formic dehydrogenase activity as this stage (Table 13).

TABLE 13.
Effect of the Ascorbic Acid System on Formate Oxidation in Seedlings.
Manometer flask contents as for Table 12.

	μl. O_2 Uptake in 40 Minutes.	Total Loss of Ascorbic Acid. (mg.)
Blank	84	—
Formate	83	—
Ascorbic acid	179	1·50
Ascorbic acid + formate	186	1·56

The increased importance of ascorbic acid oxidase in the endogenous respiration of the seedling as revealed by inhibitor studies is discussed below.

In the anaerobic experiments coupling between ascorbic acid oxidase and formic dehydrogenase was demonstrated by observing the reduction of a redox indicator by the negative system (formic dehydrogenase + formate + coenzyme I) and reoxidation of the dye by the positive system (ascorbic acid oxidase + ascorbic acid). The reducing system together with the redox dye are placed in the main part of a Thunberg tube and the components of the oxidizing system are placed in the hollow stopper. In the control tube water takes the place of ascorbic acid. After evacuation and filling with nitrogen the tubes were incubated at 38°C. until the maximum reduction of dye is obtained. Then the contents of the stopper are tipped in and if any reoxidation of the dye occurs, the time taken for a second decolorization is noted.

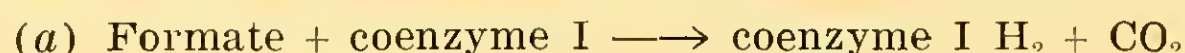
It is evident that an indicator which is either more positive than the oxidizing system or more negative than the reducing system cannot function as a carrier. Theoretically any reversible indicator whose potential lies in the range between the two enzyme systems should be able to catalyse the interaction if one is to occur. Methylene

TABLE 14.
Ascorbic Acid Oxidase–Formic Dehydrogenase Coupling in a pH 6·0 Buffer Extract of Pea Seeds.

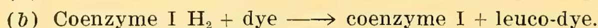
	1	2	3	4
Main tube—				
Enzyme preparation	0·5 ml.	0·5	0·5	0·5
Coenzyme I (120 μg.)	0·2 ml.	0·2	0·2	0·2
0·2 M sodium formate	0·5 ml.	0·5	0·5	0·5
0·05% redox dye	Nile blue		Methylene blue	
	0·5 ml.	0·5	0·5	0·5
Stopper—				
Enzyme preparation	0·5 ml.	0·5	0·5	0·5
Ascorbic acid (1·76 mg.)	—	0·3 ml.	—	0·3
Water	0·3 ml.	—	0·3	—
Time for first decolorization	25 mins.	25 mins.	17 mins.	17 mins.
Time for second decolorization	No colour developed	80 mins.	No colour developed	

blue and thionine, whose potentials of + 0·011 and + 0·63 respectively are more positive than the oxidizing system, were not suitable for these experiments. The carrier most suited was Nile blue, whose potential of −0·116 lies in the range between the potentials of the two enzyme systems. In all experiments with Nile blue the indicator was either partly or completely reoxidized by the ascorbic acid system before being again reduced to the leucoform. The extent of the reoxidation was found to be determined by the relative activities of the oxidizing and reducing systems as shown by control manometric experiments. The ascorbic acid and extract were mixed some time before gassing to allow for formation of the dehydroascorbic acid necessary for the reoxidation of the leuco-dye. Controls with enzyme + water in the stopper showed no trace of reoxidation of the indicator.

From gas exchange measurements the equation for the oxidation of formate by formic dehydrogenase of pea seeds has been worked out by Adler and Sreenivasaya (1937) and found to be: $HCOOH + \frac{1}{2} O_2 \longrightarrow H_2O + CO_2$. From the results in Table 14, then it seems that the entire system in such anaerobic experiments is:

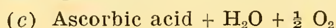


(*a*) Formate + coenzyme I $\longrightarrow$ coenzyme I H_2 + CO_2



(*b*) Coenzyme I H_2 + dye $\longrightarrow$ coenzyme I + leuco-dye.

Possibly flavoprotein is involved as carrier between coenzyme and dye in reaction (*b*).



(*c*) Ascorbic acid + H_2O + $\frac{1}{2}$ O_2

The nature of the mechanism involved in the reduction of dehydroascorbic acid has not been investigated. The close association of glutathione and ascorbic acid is seen in the observation that in germinating pea seeds these two substances appear at the same time and increase in concentration in a parallel manner (Hopkins and Morgan, 1943). In any such plant tissue the possibility that the ascorbic acid–glutathione relationships, as outlined by Hopkins and Morgan (1936), have some physiological function cannot be overlooked. That the reduction of ascorbic acid by glutathione in some plants is due to a reducing catalyst, an enzyme to which the name dehydroascorbic acid reductase has been given, is now an accepted fact and its properties, pH optimum, occurrence, etc., have been thoroughly worked out (Crook, 1941). Reducing catalysts of this type have been demonstrated in the juice of peas and beans by Kohman and Sanborn (1937), who showed that the juice of peas catalysed the reduction of dehydroascorbic acid by reduced glutathione (G.S.H.). However, as shown by Crook and Morgan (1944), the activity of the dehydroascorbic acid reductase is much lower than that of ascorbic acid oxidase so that the presence of G.S.H. does not completely protect ascorbic acid from oxidation. Some other system for the reduction of dehydroascorbic acid must therefore be operative in peas. Possibly there is a simple transfer of hydrogen from coenzyme I to dehydroascorbic acid.

Peroxidase.—In the question of ascorbic acid oxidation the presence of a specific ascorbic acid oxidase as distinct from a cytochrome catalysed oxidation, and the absence of any catechol action has left only the possibility of a peroxidase forming a terminal oxidase system with ascorbic acid.

It was found that a fresh extract from either pea seeds or seven-day seedlings would not produce any colour with 0·1 ml. of a 1% solution of guaiacol, benzidine (slight coloration) or α-naphthol. However, on the addition of 1 drop of 6% hydrogen peroxide to each tube, there was a marked positive reaction. Peroxidase then is present in both stages.

An experiment carried out by James and Cragg (1943) on barley sap was repeated with pea extract. The latter plus hydrogen peroxide will oxidize benzidine almost immediately. If ascorbic acid is added there is a lag period until the oxidation of the ascorbic acid by the quinone formed (p-quinone di-imide) is complete. Then the blue

colour of the oxidized benzidine suddenly appears. As in James' experiment, it was found with pea extract that the length of the lag was proportional to the amount of ascorbic acid added. From this it seems inevitable that, if hydrogen peroxide is formed in the pea seed, the peroxidase system should co-operate in the oxidation of ascorbic acid. However, the absence of H_2O_2 in pea extract is shown in experiments quoted above which showed peroxidase activity only when H_2O_2 from an external source was added. Also it is doubtful whether enough H_2O_2 can occur in cells which, like those of the pea, contain a strongly active catalase; and whether sufficient phenols are present is also somewhat unlikely.

Further, 0·001 M cyanide almost completely inhibits ascorbic acid oxidation in a pH 6·4 buffer extract of pea seeds. At this concentration the inhibition of peroxidase is far from complete, so that peroxidase cannot be responsible for the oxidation of ascorbic acid to the exclusion of a direct ascorbic acid oxidase.

What the value of peroxidase is to the plant is still an open question. On the whole it seems extremely unlikely that peroxidase should combine with ascorbic acid in forming a terminal oxidase system.

Inhibitor Studies.

Some ideas on the relative importance of the various oxidation mechanisms in the respiration of the pea seed and seedling can be obtained from studies using specific inhibitors. The following experiments on the effect of inhibitors on endogenous respiration were all carried out at the normal reaction of pea extract, pH 6·4. The enzyme preparation is an untreated water homogenate centrifuged for 10 minutes. Manometer flasks contained 1·9 ml. of this supernatant, 0·8 ml. 0·2 M phosphate buffer pH 6·4, 0·2 ml. 20% NaOH in the centre well. In the side bulb 0·3 ml. water or 0·3 ml. inhibitor was placed. All inhibitors were neutralized before use and made up to their final concentration in phosphate buffer pH 6·4. Oxygen uptake was measured over a period of 60 minutes and all results are given as percentage inhibition caused by the inhibitor. Under the conditions set down above the oxygen uptake for seed preparation in 60 minutes is 42–55 μl., and for a seedling preparation, 50–67 μl.

Cyanide.—Both the cytochrome system and the ascorbic acid system are sensitive to small concentrations of cyanide. The inhibitor is, therefore, by no means specific but its effects are interesting in determining the activity of the respiratory system resistant to poisoning with dilute cyanide. One cannot conclude at present that such a fraction of the respiratory oxygen uptake is necessarily operated by flavoprotein systems. However, coupled with the observation that the flavoprotein content of peas rises on germination (after two days it increases to three times its original value; van Herk, 1935) the fact that cyanide inhibition is less in the seedling (Table 15) makes the flavoprotein hypothesis extremely likely.

Table 15.
Inhibition Produced by Cyanide on Seeds and Seedlings.

Cyanide (Final Concentration).	Percentage Inhibition of Control.	
	Seeds.	Seedlings.
1×10^{-2} M	58	25
5×10^{-3} M	52	24
1×10^{-3} M	45	18
5×10^{-4} M	39	15
1×10^{-4} M	31	11
1×10^{-5} M	22	9

Another point to be considered here is that no serious inactivation of dehydrogenases is obtained with cyanide concentrations of less than 0·002 M. The one exception

to this, however, is formic dehydrogenase, which is inhibited by concentrations of $1 \cdot 3 \times 10^{-3}$ M. The fact that formic dehydrogenase activity decreases in the seedling is in agreement with the decrease in cyanide inhibition in the seedling.

In experiments on cyanide loss of concentration of the inhibitor by distillation into the alkali of the centre cup was prevented by using cyanide-alkali mixture (Krebs, 1935).

Azide.—This inhibitor is specific in that it poisons the cytochrome system but not the ascorbic acid system (James, 1946). The results obtained with various concentrations of azide are summarized in Table 16.

TABLE 16.
Inhibition Produced by Azide on Seeds and Seedlings.

Azide Concentration.	Percentage Inhibition.	
	Seeds.	Seedlings.
10^{-2} M	53	38
10^{-3} M	43	35
10^{-4} M	37	26
10^{-5} M	21	12

Diethyldithiocarbamate.—Ascorbic acid oxidase is strongly inhibited by this copper precipitant but cytochrome oxidase only slightly (James, 1946). Coupled with the results obtained with azide then the figures in Table 17 can give some information on the relative activity of these two terminal oxidases in seed and seedling. It appears that azide inhibition is greater in the seedling whereas diethyldithiocarbamate is more effective in inhibitng the oxygen uptake of the seed extract. These results can be correlated with those reported earlier in this paper, namely, the increased ascorbic acid oxidase activity on germination and the decreased effect of cytochrome on seedling respiration. It seems very probable then that while these two terminal oxidases are of more or equal value to the economy of the seed, the ascorbic acid system becomes the more important in the seedling.

TABLE 17.
Inhibition Produced by Diethyldithiocarbamate on Seeds and Seedlings.

Diethyldithiocarbamate Concentration.	Percentage Inhibition.	
	Seeds.	Seedlings.
10^{-3} M	31	40
10^{-4} M	25	36
10^{-5} M	19	28

Iodoacetic Acid.—Triosephosphate accumulates in iodoacetate-poisoned barley digests containing hexosediphosphate, and the acid is known to inhibit also the alcohol dehydrogenase of oat seedlings (Berger and Avery, 1943). James (1946, p. 429) and Turner (1937) agree in attributing the sensitivity of plant respiration to iodoacetate poisoning to an inhibition of the triosis stage of glycolysis. Table 18 shows the inhibition produced by iodoacetate on pea seeds and seedlings.

The increased inhibition on germination can be correlated with the fact that the C_4 acids and citrate have been found to exert more effect in the seedling. Albaum and Eichel (1943) found similar effects in the oat seedling where iodoacetate inhibition and C_4 acid stimulation could be demonstrated only after 72 hours of growth. They were

TABLE 18.
Inhibition Produced by Iodoacetate on Seeds and Seedlings.

Iodoacetate Concentration.	Percentage Inhibition.	
	Seeds.	Seedlings.
10^{-2} M	38	58
10^{-3} M	21	43
10^{-4} M	14	30
10^{-5} M	11	22

able to conclude from this and other evidence that fat is metabolized mainly in the early stages and that sugar is the main metabolite after 72 hours. However, pea seeds have very little fat, none of which is storage material but only phosphatides concerned in the cell and nuclear structure. Also, in the seedling, pyruvate and the dicarboxylic acids should produce a reversal of iodoacetate inhibition if this substance is poisoning the triosis stage of glycolysis. Malate and glucose were found to overcome this effect slightly but certainly not when used in catalytic amounts. Succinate and pyruvate were entirely without effect.

It is impossible to conclude then that iodoacetate is inhibiting only the "triosis stage of glycolysis" in peas or to make any suggestions as to the type of respiratory substrate being used either before or after germination.

Malonate.—This acid is known to inhibit slightly succinic dehydrogenase and has been used to elucidate the relations between the C_4 acids making up what is known as the Szent-Györgyi cycle. Neither in the pea seed nor seedling could any significant inhibition be obtained with malonate, however, the results being given in Table 19.

TABLE 19.
Inhibition Produced by Malonate on Seeds and Seedlings.

Malonate Concentration.	Percentage Inhibition.	
	Seeds.	Seedlings.
10^{-2} M	9	10
10^{-3} M	8	5
10^{-4} M	5	7
10^{-5} M	4	3

The whole question of the significance of malonate inhibition in plants is at present uncertain, but it seems clear from these results that no C_4 acid system in the sense of the Szent-Györgyi cycle is operating here.

Semicarbazide.—This substance has an interesting effect on the endogenous respiration and alcohol oxidation of pea seeds and seedlings as shown in Table 20.

These results show that seedlings contain much less active alcohol dehydrogenase than seeds and also the effect of semicarbazide is quite different in the two materials. In the seed alcohol oxidation is greatly accelerated by semicarbazide presumably because of its binding effect on the reaction product, acetaldehyde. Endogenous respiration in the seed is also accelerated by semicarbazide. In the seven-day seedling the substance was found to inhibit endogenous respiration and, to a lesser extent, alcohol oxidation. These effects suggest that endogenous respiration in seeds is partly due to alcohol dehydrogenase whereas in the seedling it is not.

TABLE 20.

Effect of Semicarbazide on the Endogenous Respiration and Alcohol Oxidation of Pea Seeds and Seedlings.

Manometer flask contents: 1·0 ml. untreated water extract, 0·8 ml. 0·2 M phosphate buffer pH 7·9, 0·3 ml. coenzyme I (90 µg.), 0·1 0·5% methylene blue (one side bulb), 0·3 ml. 3·2 M ethyl alcohol, and 0·5 ml. 0·25 M semicarbazide solution as indicated. Water to 3·2 ml.

Addition.	µl. O_2 Uptake in 90 Minutes.	
	Seed.	Seedling.
Blank	100	65
+Semicarbazide	165	47
Alcohol	133	75
Alcohol+semicarbazide	337	62

In support of this hypothesis of an important role for alcohol dehydrogenase in the endogenous respiration of the seed we find that pyruvic decarboxylase activity which is extremely high in the seed falls off with germination (Tables 21, 22).

TABLE 21.

Carboxylase Activity in the Seed.

Flask contents: 1·4 ml. pea extract, 0·8 ml. 0·2 M phosphate buffer pH 6·85, 0·1 ml. 0·5% methylene blue, 0·3 ml. 0·03 M pyruvate or 0·3 ml. water in the side arm. R.Q. was determined by the direct method outlined in Umbreit, Burris and Stauffer (1945, p. 16) over a period of 60 minutes.

	O_2 Uptake.	CO_2 Output.	R.Q.
Blank	40 µl.	66 µl.	1·66
Pyruvate	25 µl.	275 µl.	10·83

TABLE 22.

Carboxylase Activity in Seven Day Old Pea Seedlings.

Manometer contents as in Table 21. These results are for an 80-minute period.

	O_2 Uptake.	CO_2 Output.	R.Q.
Blank	107 µl.	166 µl.	1·55
Pyruvate	96 µl.	197 µl.	2·03

With the seeds 96% of the pyruvate added was decarboxylated in 60 minutes, whereas in the seedling extract only 14% was decarboxylated in 80 minutes. This decline of a reaction which seems the most likely source of alcohol, at a time when there is a parallel decline of alcohol dehydrogenase, points to a probable use for the dehydrogenase in the economy of the seed.

Finally, in discussing the place of alcohol dehydrogenase in the seed, it can be recorded here that the endogenous respiration of the seed is accelerated by the addition of small amounts of coenzyme I whilst in the seven-day seedling the effect is less marked (Table 23).

Van Herk (1935) points out that the cozymase of germinating peas is initially large and remains so for four days, but after seven days it has fallen to one-fifth or one-sixth of its original value. These observations make it extremely likely that much of the

TABLE 23.

Effect of Coenzyme I on Endogenous Respiration.

Flask contents: 1·6 ml. untreated water extract of peas, 0·8 ml. 0·2 M phosphate buffer pH 6·4, coenzyme I added from side arm (30µg.–180µg.), 0·2 ml. 20% NaOH in centre well.

Amount of Coenzyme I Added.	µl. O_2 Uptake in 120 Minutes.	
	Seeds.	Seedlings.
—	50	181
30µg.	53	182
60µg.	58	185
120µg.	65	186
180µg.	76	188

dehydrogenation in the seed depends on coenzyme I-requiring dehydrogenases and less so in the seven-day seedling. The only plant dehydrogenases known to need coenzyme I are alcohol, formic, lactic, malic, glutamic, β-hydroxybutyric and triose-phosphoric dehydrogenases. The first five have been studied in peas and only alcohol and formic dehydrogenase have been found to fall off in activity during germination. The difficuluty in the way of proving formic dehydrogenase as a normal link in biological oxidation in the pea is that there is no specific inhibitor of the enzyme which, by application to the endogenous respiration, can prove a similar inhibition. With alcohol dehydrogenase, however, there is the supporting evidence of the semicarbazide and carboxylase experiments quoted above.

In the seed, then, alcohol dehydrogenase and possibly formic dehydrogenase are the main enzymes concerned in the first or dehydrogenating step of biological oxidation.

DISCUSSION.

On examining the results obtained with inhibitors in seeds and seedlings some correlation can be obtained with the increase or decrease of certain enzymic activities with growth. The results obtained have been tabulated below (Table 24).

TABLE 24.

Correlation of Results Obtained with Inhibitors in Seeds and Seedlings with the Increase or Decrease of Certain Enzymic Activities with Growth.

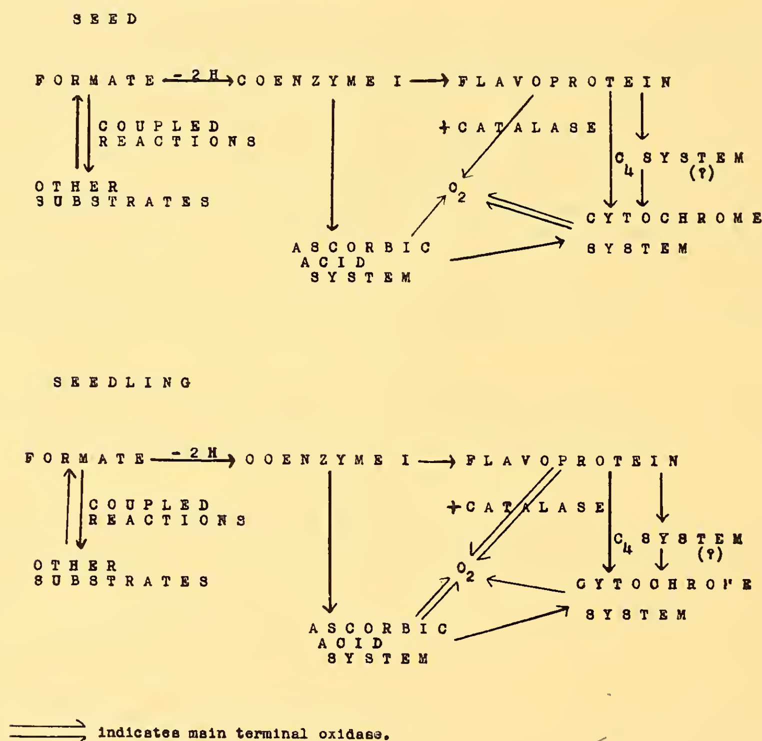
Hydrogen Carriers.	Inhibitors.
I. *Seeds*—	
Ascorbic acid oxidase present.	10^{-3} M diethyldithiocarbamate gives 31% inhibition.
Cytochrome system operative.	10^{-3} M cyanide—45%.
Alcohol and formic dehydrogenases active.	10^{-3} M azide—43%.
C_4 acid system—no activity.	10^{-3} M iodoacetate—21%.
High carboxylase activity.	10^{-3} M malonate gives no inhibition (2%).
Added coenzyme I has marked effect on endogenous oxygen uptake.	Semicarbazide gives 63% acceleration of endogenous respiration.
II. *Seedlings*—	
More ascorbic acid oxidase activity.	Diethyldithiocarbamate inhibition rises to 40%.
Less cytochrome effect.	Cyanide and azide inhibition fall to 18% and 35%
Alcohol dehydrogenase almost absent.	No malonate inhibition.
Formic dehydrogenase less active.	Iodoacetate inhibition rises to 43%.
Flavoprotein effective in formate oxidation.	Semicarbazide inhibits by 28%.
C_4 acids slightly more effective.	
Low carboxylase activity.	
Added coenzyme I has less effect on endogenous O_2 uptake.	

It is difficult to interpret the effects of poisons on such complex processes as oxidation since so many consecutive reactions are involved. However, coupled with the information obtained on the activity of the hydrogen carriers the following general conclusions can be stated.

1. Based on the coenzyme activation of the respiration of seed extract and the degree of activity of the various dehydrogenases in seed and seedling, it seems likely that in the seed alcohol dehydrogenase and formic dehydrogenase are the main enzymes concerned in the first or dehydrogenating step of biological oxidation. This is supported in the case of alcohol dehydrogenase by the falling off of carboxylase activity in the seedling, a reaction which seems the most likely source of alcohol and the acceleration of seed respiration but not that of seedlings by semicarbazide.

2. Formic dehydrogenase is capable of coupling with other dehydrogenases.

3. Development of both iodoacetate inhibition and C_4 acid utilization with germination indicate the utilization of sugar as respiratory substrate only after about 72 hours. However, this could not be confirmed by demonstrating a removal of iodoacetate inhibition on the addition of C_4 acids and pyruvate.



SCHEME II

4. Lack of malonate inhibition and of noticeable effects on addition of catalytic amounts of C_4 acids make the operation of the Szent-Györgyi cycle extremely improbable. However, formate oxidation is accelerated by two of these acids, malate and fumarate and also citrate so that they may be of some importance in biological oxidation.

5. The main terminal oxidase in the seed is cytochrome oxidase. With germination this activity falls off and the activity of ascorbic acid oxidase, already of importance in the seed, increases. Finally, after seven days, most of the cyanide sensitive respiration can be accounted for by the oxygen consumption of the ascorbic acid system. Formate oxidation has been shown to be coupled with both cytochrome and ascorbic acid systems in seed and seedling.

6. With regard to the cyanide insensitive respiration which seems to amount to a fairly high percentage (41% in the seed, 75% in the seedling), this is in all probability due to a flavoprotein system since there is a corresponding rise in flavoprotein content on germination. Also formate oxidation is accelerated by flavoprotein, especially in the seedling.

Some conclusions on the oxidation mechanisms of the seed and seven-day seedling, and the place of formic dehydrogenase therein, can now be made. The paths used in the breakdown of formate have been narrowed down and, in place of Scheme I, the following diagrams give mechanisms for the oxidation of this substrate, based on the results of experiments quoted above (Scheme II).

Summary.

1. Various oxidation mechanisms of the green pea seed and seedling and their relation to formic dehydrogenase have been studied. These include other dehydrogenases, intermediate hydrogen carriers such as flavoprotein and the dicarboxylic acids and terminal oxidases.

2. As a result of these studies a normal role in the first or dehydrogenation step of biological oxidation can be assigned to formic dehydrogenase.

3. Inhibitor studies on seed and seedling extract have revealed some significant changes in enzyme activity on germination.

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